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RESEARCH ARTICLE

Water acidification causes death of marine ornamental fish (Perciformes: Pomacentridae) during transport: contributing to the conservation of wild populations

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ABSTRACT. Pomacentridae is a common family in the aquarium fish trade. Most species are harvested from nature. Here we evaluate the following water parameters in the pomacentrid sergeant major, *Abudefduf saxatilis* (Linnaeus, 1758), to assess their stress level during a 24, 48, and 72 hours transport: dissolved oxygen (DO), total ammonia, and pH. In addition, we evaluated the following physiological parameters: plasma osmolality, muscle water content, blood glucose, and the enzyme activities of the branchial carbonic anhydrase (CA), the hepatic glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD). The mortality of fish measuring >6 cm total length was 22%, while no mortality was observed for fish measuring <6 cm. The pH of the water was significantly correlated with fish mortality, especially for the initial 24 hours of transport. Hypoxia after 24–48 hours also led to fish mortality, but build up ammonia was not a problem even after 72 hours. We suggest that a minimum water volume of 125 ml/g fish is necessary for safe and cost-effective transport of the sergeant major, preferably with <6 cm in total length.

KEY WORDS. Ammonia, aquarium trade, carbon dioxide, fish conservation, pH.

INTRODUCTION

The trade of marine ornamental fish (MOF) is essentially based on harvesting fish from nature. Fish are mostly caught from tropical countries and exported to developed nations (Wood 2001b, Wabnitz et al. 2003, Shuman et al. 2004, Alencastro et al. 2005). Intense and repeated harvesting from nature, which is magnified by unsuccessful transport events, may cause a problem to the conservation of the target species. If most fish survived their journeys to their final destinations, there would be less specimens captured, but in order to guarantee higher survival rates during transport, it is necessary to minimize the stress on fish, from capture to their target environment (Wood 2001b, Wabnitz et al. 2003). Despite all technology available for transport, however, the variables associated with fish survival during transport are not fully understood.

The conservation of MOF, or the assurance of sustainability in the MOF trade, has been extensively discussed in the literature: management strategies (Wood 2001a, b, Wabnitz et al. 2003, Alencastro et al. 2005), improvement of law enforcement (Gasparini et al. 2005, Sampaio and Ostrensky 2013, Sampaio et al. 2015), invasion by non-native species (Padilla and Williams 2004, Semmens et al. 2004, Weigle et al. 2005), or the need for some species to be reared in aquaculture (Job 2005). However, research describing the association between the stress of capture and transport, and its relationship with the conservation of MOFs, has not been found.

Transport of live fish is a crucial process for the aquarium business. Successful transport is an important step to minimize fish mortality (Wood 2001b, Wabnitz et al. 2003, Alencastro et al. 2005). Ornamental fish caught in the wild are frequently

transported over very long distances (Brinn et al. 2012). For example, fish collected in the Philippines exported to the United States travel for about 20–25 hours until they arrive in Los Angeles, and more than 40 hours until they are in New York. Depending on the conditions of collection and transport, high mortality (90%) may occur (Rubec et al. 2001).

Live fish need specific handling protocols for effective transport, and protocols vary according to how fish tolerate the changes that occur in the water during transport (Treasurer 2012). Since data on ornamental fish tolerance to stress is very scarce, these protocols are usually unavailable. These protocols need to take into account the loss of water quality and the arousal of physiological disturbance to fish during transport.

Physiological tolerance to a decrease in water quality and temperature stress during transport are not well documented for MOF (Chow et al. 1994, Paterson et al. 2003, Hur et al. 2007). Among the physiological data available in the literature, the following research has been conducted and is relevant to our paper: the physiological response (oxygen consumption rate and excretion of metabolic wastes) of the clownfish *Amphiprion ocellaris* Cuvier, 1830 (Pomacentridae) to transport (Chow et al. 1994), the evaluation of transport and handling of the seahorse *Hippocampus abdominalis* Lesson, 1827 on blood glucose, cortisol and lactate (Wright et al. 2007), and the use of the essential oil of *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson (Plantae: Verbenaceae) as an anesthetic for the seahorse *Hippocampus reidi* Ginsburg, 1933, evaluating blood glucose and leucocyte count (Cunha et al. 2011).

It is generally understood that fish health and survival during post-transport is associated with the maintenance of water quality, therefore transport protocols that ensure good water quality should be part of the MOF industry goals (Bruckner 2005). Many studies focus on the use of anesthetics (Tondolo et al. 2013, Parodi et al. 2014, Zeppenfeld et al. 2014), or on ideal fish density inside the transport containers (Carneiro and Urbinati 2002, Urbinati et al. 2004, Tenningen et al. 2012). The evaluation of stress-associated responses of fish to transport have mostly focused on physiological stress markers, such as cortisol and glucose (Schreck et al. 1995, Sampaio and Freire 2016).

The ongoing/growing interest in the MOF trade has led to increased capture pressures on wild fish. This study evaluated physiological and biochemical responses (plasma osmolality, glucose, muscle hydration, and oxidative stress enzymes) of the sergeant major fish *Abudefduf saxatilis* (Linnaeus, 1758), and changes in the water of transport (dissolved oxygen, pH, and ammonia) in a situation simulating long transport. *Abudefduf saxatilis* belongs to Pomacentridae, one of the most relevant families in the worldwide MOF trade (Wood 2001b, Wabnitz et al. 2003). Fish of the genus *Abudefduf* are among the 10 most-exported species worldwide and the most imported in the United States (Wabnitz et al. 2003). The species chosen is widespread along the Brazilian coast (Atlantic sergeant) with its aesthetically attractive appearance as potential commercial relevance as an ornamental fish. The ultimate goal of this study is to foster studies on the successful transport of marine ornamental fish either from the wild or from cultured specimens, thus contributing to the conservation of their stocks in nature.

MATERIAL AND METHODS

Fish were caught in the state of Santa Catarina, city of Bombinhas, Sepultura Beach (27°08'28"S, 48°28'42"W), Brazil, between October 28th, 2013 and April 9th, 2014, using a handmade net. The total length of fish varied between 3.4 and 18.2 cm, mean ± SEM of 6.52 ± 0.31 cm. This size variability reflected the fact that they were sampled in nature. After being captured, the animals were individually placed in plastic bags of low-density polyethylene (LDPE), containing ~1/3 (volume) of seawater. The salinity of the seawater used to fill the bags was always of 35 psu, and temperature was of 21.2–25.6 °C. Two sizes of plastic bags were used (small: 12 cm X 33 cm, and large: 24 cm X 34 cm, both 0.2 mm-thick), and were chosen according to the size of the fish, in order to attenuate variability in the proportion of water volume to the weight of the fish. Achieving a precise proportion between fish weight and water volume inside the bags was not attempted here. The goal was to simulate normal catch conditions of wild fish. Each plastic bag was filled up with air of 100% oxygen to ~2/3 of its volume, allowing space only for closing the bag. The mouth of the bag was then twisted and tightly closed with several rounds of rubber bands. Bags were placed inside Styrofoam boxes, which were transported by car (4–5 h trip) to the Laboratory of Comparative Physiology of Osmoregulation in Curitiba. Upon arrival in the laboratory, the boxes were placed on the lab floor and kept closed. Fish were periodically (~every 12 hours) checked for mortality. Dead fish were sampled immediately, and this occurred in <24 h and between 24 and 48 h. After 48 h there was no further mortality. The final "n" for each group was: 16 live and 9 dead (≤24 h); 13 live and 5 dead (24–48 h); and 13 live fish (72 h).

Plastic bags were opened (total n = 56, 42 live and 14 dead) at the set times, and dissolved oxygen (DO), pH and temperature (21.4–26.0 °C) were immediately measured. Dissolved oxygen (DO), ammonia ($N\text{-}NH_3$), and pH were measured in the water of each sampled bag, including those that had dead fish, immediately after removal of the fish. Ammonia was measured using commercial kits (AlphaKit, Brazil) with absorbance read at 630 nm (spectrophotometer ULTROSPEC 2100 pro – Amersham Pharmacia Biotech, Sweden). DO and pH were respectively measured using a dissolved oxygen meter (Lutron, model DO-5519, Brazil) and a portable pH meter (pHtek, model PH100, Brazil).

After 24, 48 or 72 h of transport confinement, live fish were anaesthetized with benzocaine (80 mg/L seawater) in 2-liter plastic vials containing seawater. Within 2–3 min, fish were in complete anesthesia. Fish were measured for total length, weighed, and had a blood sample taken from the caudal vein using heparinized syringes. They were then euthanized by spinal cord section. A drop of blood was used to measure blood glucose

(human glucotest Accu-Check®-Roche-Performa Nano model), and the remaining volume was centrifuged for 5 min at 2,100 xg to allow for plasma separation. Plasma samples were kept in the freezer at -20 °C for the osmolality assay. Tissues (gills, liver and axial muscle fragments) were removed and immediately frozen at -80 °C for posterior assays of enzyme activities. The experimental procedure described here was approved by the Committee on Ethics and Animal Welfare of the Federal University of Paraná (certificate #761/2014, issued on February 13, 2014).

An additional group of fish (n = 11) was collected and brought to the laboratory exactly as described above and transferred to a stock aquarium (20 L) in the lab. These specimens were kept for seven days in this aquarium and served as a reference (control) for the experimental groups that underwent 24, 48, and 72 h transport. This was performed in duplicate, with three fish in the first trial, and eight in the second trial. We decided to do this after considering that capturing/handling fish on the beach cause a certain level of stress to these animals, and since they were already stressed by then, they would could not be used for reliable physiological reference. Within a few hours (2–4 hours) in the stock tank control, fish recovered from the stress of handling, resuming a regular swimming pattern, feeding, and displaying their usual bright colors. During the maintenance period in the lab, fish were fed commercial fish flakes once a day. After this period, they were processed exactly as described for the experimental animals.

Plasma osmolality was read in undiluted samples using a vapor pressure osmometer (Wescor VAPRO 5520, USA). The percentage of water in muscle fragments was determined as the 24 h weight loss at 60 °C. Muscle slices were thawed and weighed (wet weight precision 0.1 mg, BioPar S22ST, Brazil), dried and weighed again (dry weight). The loss in weight was expressed as a percentage of the initial wet weight of the muscle slice.

Gill samples were weighed and homogenized in 10% w/v (weight/volume) with 10 mM phosphate buffer, pH 7.4 for the carbonic anhydrase (CA) assay. The homogenate was centrifuged at ~2,000 xg (5 min, room temperature) to precipitate cellular debris. The supernatant was separated. CA activity was quantified by the addition of the supernatant and distilled water saturated with CO_2 into a reaction medium containing mannitol (225 mM), sucrose (75 mM), and Tris-phosphate (10 mM), at pH 7.4. Immediately after the addition of the sample, the pH of the reaction vial was noted, every 4 s, for a total of 20 s (pH meter inoLAB benchtop pH Level 1 WTW®, Germany). Assay temperature was maintained at ~2.5 °C through an ice bath. The slope of the linear regression of pH versus time was the catalyzed reaction rate (CR) for the sample. The non-catalyzed rate of reaction (NCR, blank) was the slope of pH drop (same procedure used for the sample CR determinations) versus time using the buffer without the addition of tissue homogenate. The NCR was determined as the mean of three replicates, one taken at the beginning of the assay section, one every five sample readings, and another at the end of the session.

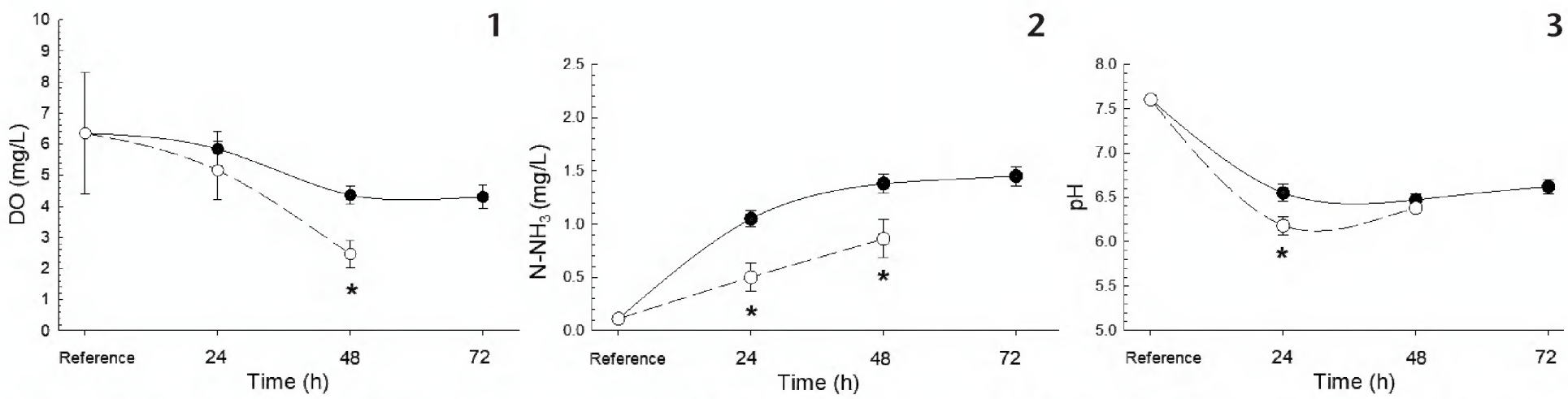
Glutathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) activities were assayed in liver samples, homogenized in phosphate buffer (0.1 M, pH 7.0), and centrifuged at 15,000 xg for 30 min, at 4 °C. Aliquots of the supernatant were separated for each enzyme assay. GST activity was quantified using 30 µl of the supernatant and 180 µl of the reaction medium containing 10 mM reduced glutathione (GSH), 60 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 0.1M potassium phosphate buffer, at pH 6.5. Activities were read at 340 nm for 180 sec at 15 sec intervals (Keen et al. 1976). CAT activity was quantified according to the method in Aebi (Aebi 1984). A volume of 30 µL of supernatant was mixed to 990 µL of a reaction medium containing 30% H_2O_2, 0.5m Methylene-diamine-tetraacetic acid (EDTA), and 1 M Tris-base at pH 8.0. Activity was proportional to the reduction in H_2O_2 absorbance after 60 s at 240 nm. SOD activity was determined as the rate of inhibition of auto-oxidation of pyrogallic acid (Gao et al. 1998) at 440 nm. One unit of SOD was defined as the enzymatic activity necessary to inhibit the reduction of NBT by 50%. Total protein concentration for all enzyme assays were determined by the Bradford method (Bradford 1976) with bovine serum albumin as a standard, and expressed in $mg.mL^{-1}$, in triplicates. GST and CAT were expressed as µmol $(min.mg\ protein)^{-1}$ and SOD as $U.mg\ protein^{-1}$.

One-way ANOVA was used to compare the reference group with the experimental time groups (24, 48 and 72 h). Normality and homogeneity of variances among groups were tested using the Shapiro-Wilk test. The Kruskal-Wallis test was used for data that did not meet the requirements for normality. The results are expressed as the mean ± SEM for parametric data, and median (25–75% quartiles) for non-parametric data (Table 1). Student's t-tests (or Mann-Whitney Rank Sum Test, when normality failed) were employed for comparisons between live and dead fish (Figs 1–3). Pearson correlation was used to verify the association between variables. Analyses were performed using the SigmaPlot® 11.0 software, and the significance level was always set at 0.05.

RESULTS

Fish mortality during the transport experiments

The total number of fish used in this study was of 67, but 14 individuals (21%) died before being processed (nine before 24 h, and five between 24 and 48 h), inside the individual plastic bags. The length of live fish ranged between 3.4 and 8.1cm (0.75–8.52 g), and there was one exceptionally large fish measuring 14.2 cm (54 g). The mean size of live fish was 5.49 ± 0.14 cm, and 3.01 ± 0.25 g (mean ± SEM, without the outlier of 14.2 cm). Dead fish ranged between 6.4 and 18.2 cm long (4.5–125 g), with a mean size of 9.48 ± 0.93 cm, and weight of 23.28 ± 6.92 g. Observations of raw mortality data versus size promptly indicated that weight increased much faster than body length (Fig. 4). Furthermore, larger fish died in greater numbers. The weight x length graph clearly showed a steep rise in weight



Figures 1–3. Time course of variation of water parameters for living and dead *A. saxatilis*: (1) water dissolved oxygen (DO, mg/L), (2) total ammonia-N (NH_3-N, mg/L) and (3) pH versus time (h). Values shown are means ± SEMs. When not apparent, SEM is smaller than the symbol. This happened for the water of the reference fish, ammonia and pH. Black circles: fish that were alive at the end of the experiment; white circles: fish that died during the experiment. *: water parameter for dead fish is different from that of live fish.

Table 1. Physiological parameters of live *Abudefduf saxatilis* of the reference and experimental groups maintained for 24, 48, or 72 hours in the plastic bags. Data are mean ± SEM or median* [25–75% quartiles](n). No significant differences were detected among groups, except for SOD (lower case letters).

Parameter	Reference	24 hours	48 hours	72 hours
Plasma osmolality	232±16	271±9	243±16	244±1
(mOsm/kg H_2O)	(7)	(7)	(10)	(6)
Glycemia*	51.0 [47.5–64.5]	76.0 [51.0–291.0]	53.5 [38.5–182.5]	41.0 [37.5–79.5]
(mg/dL)	(7)	(7)	(10)	(6)
Muscle water content*	79.0 [78.3–79.4]	78.3 [77.5–79.7]	78.2 [77.5–78.8]	78.4 [78.0–78.9]
(%)	(11)	(13)	(13)	(12)
Gill Carbonic Anhydrase*	25.7 [20.2–36.1]	21.7 [18.4–30.4]	20.2 [15.7–22.8]	19.7 [16.1–22.6]
(/mg protein)	(8)	(12)	(10)	(10)
Liver GST	199±20.2	223±12.8	221±14.4	222±13.8
(µmol/min.mg protein)	(5)	(10)	(8)	(9)
Liver CAT*	53.4 [39.8–89.6]	50.1 [45.9–75.2]	55.5 [50.7–59.7]	45.7 [36.3–64.9]
(µmol/min.mg protein)	(5)	(10)	(7)	(8)
Liver SOD	127.3±16.9[a]	66.2±6.7[b]	81.5±3.7[b]	67.1±4.6[b]
(U/mg protein)	(6)	(9)	(7)	(8)

for fish longer than 6–8 cm, which is associated with a high mortality rate – with the sole exception of the large 14.2 cm specimen, which survived the 24 h transport. When separate (live or dead fish) regression lines are fitted to the weight/length versus length data, the slope of the lines change from 0.27 (live) to 0.50 (dead fish, Fig. 5).

The ratio of bag water volume to the weight of *A. saxatilis* was variable especially for small fish, following a hyperbolic shape against fish length (Fig. 6). It was possible to see that the large dead fish had a much lower ratio of bag water volume to fish weight (Mann-Whitney $p < 0.001$) than the small live fish.

Water parameters changing upon transport

Dissolved oxygen (DO) did not decrease in the water containing live fish but decreased in the packages with dead fish between 24–48 h, when compared to the water containing fish that survived the 48 h transport (Fig. 1). Total ammonia in the water was greater after 24 and 48 h for live fish, when compared to the water containing dead fish (Fig. 2). The pH of the water in the bags with dead fish was lower after 24 h when compared to the pH of the water with live fish after the same amount of time, 24 h (Fig. 3).

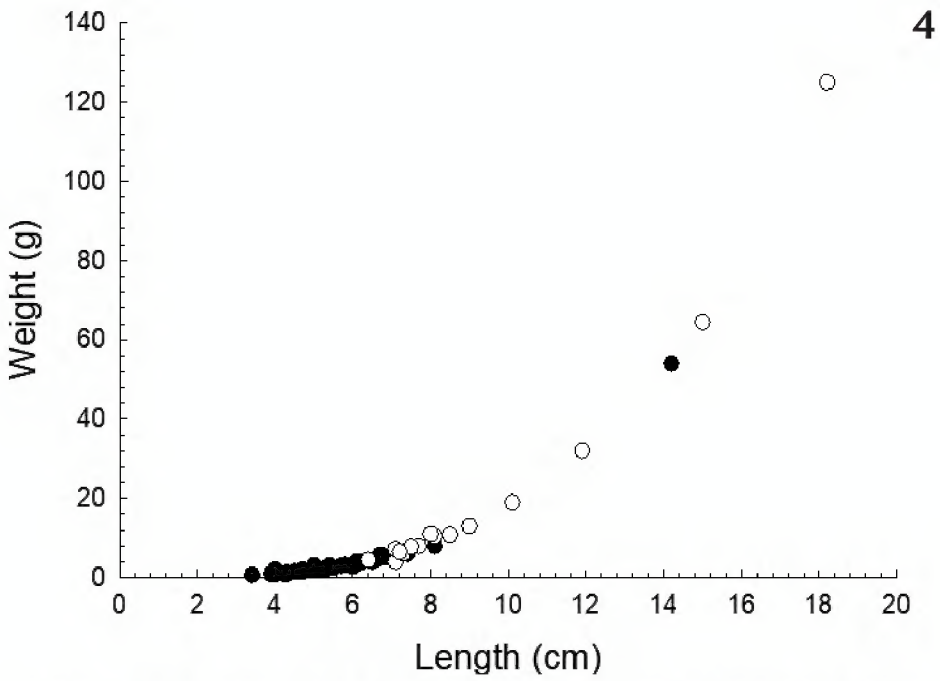
Physiological parameters

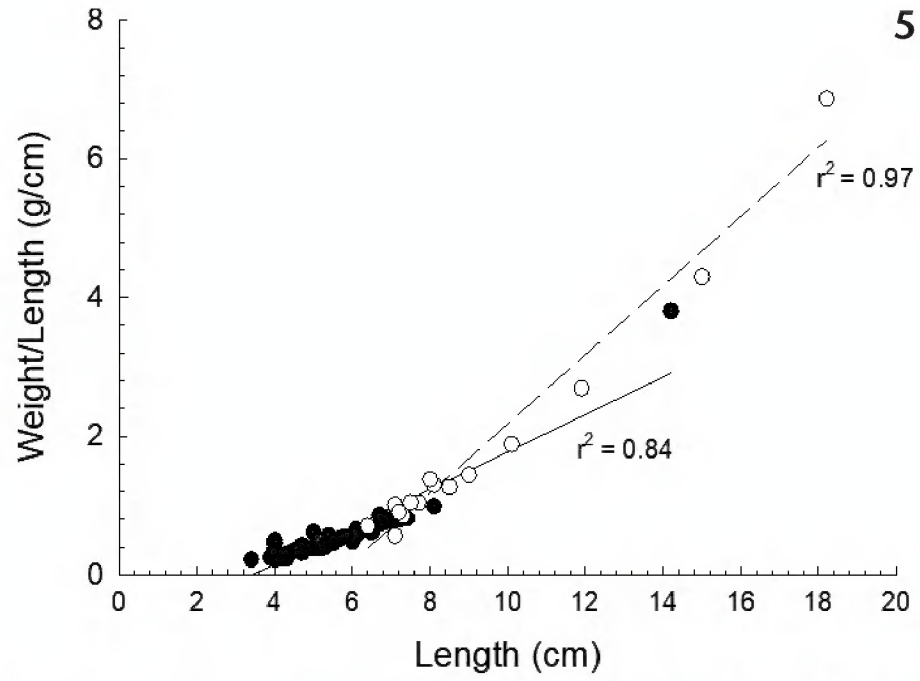
The physiological parameters remained unchanged in live fish, in all transport groups (reference, 24, 48, 72 h, Table 1). The only significant difference was noted in the SOD activity of the liver, with transported fish showing lower values than the fish from the reference group (Table 1).

DISCUSSION

Fish mortality during the transportation experiments

The results show a very conspicuous change in the pattern of increase in mass/weight, as fish grew in length. This can be easily observed when separate regression lines (one for live, an-





Figures 4–5. Relationship between weight (g) and total length (cm) of *A. saxatilis*: (4) weight (g) as a function of the total length (cm) of the fish. Black circles, fish that were alive at the end of the experiment (n = 53); white circles, fish that died during the experiment (n = 14). (5) Weight/length ratio versus fish length, with separate linear regressions for living (solid line) and dead (dashed line) fish, and respective r^2 values.

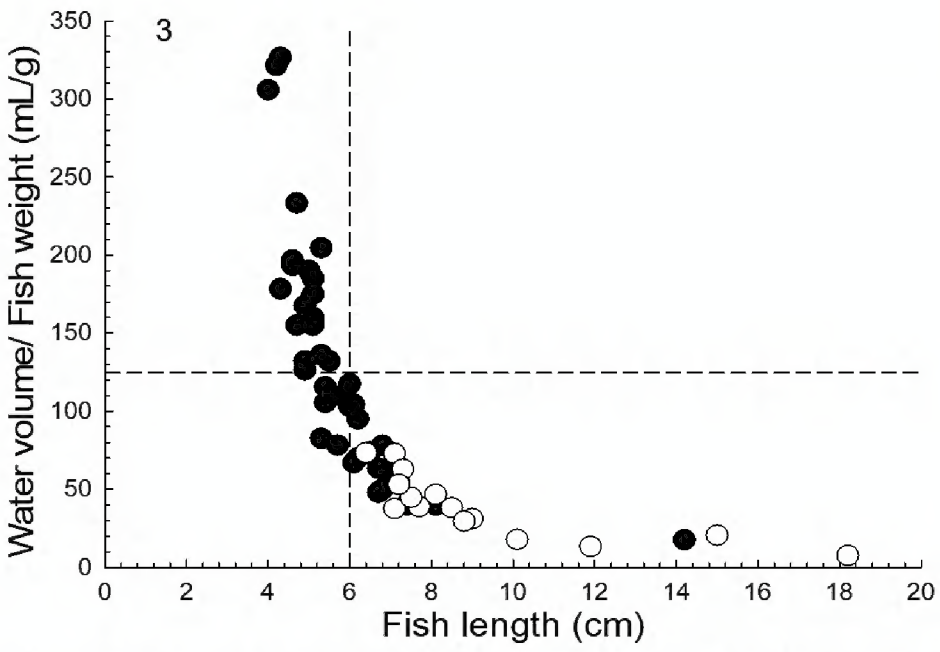


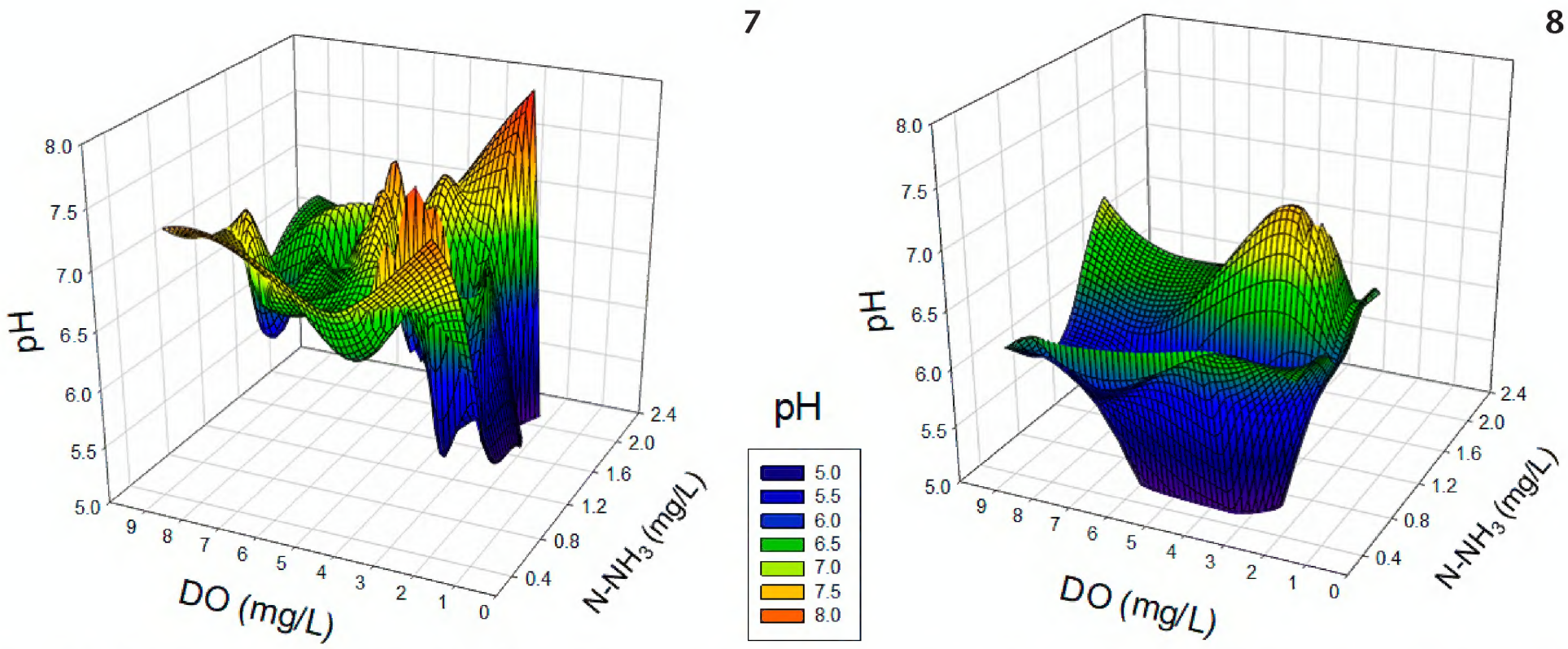
Figure 6. Transport water volume (ml) per gram of fish weight versus total body length (cm). Black circles, fish that were alive at the end of the experiment; white circles, fish that died during the experiment. Vertical and horizontal dashed lines respectively indicate maximum suggested length for the transport of *A. saxatilis* (6 cm) to ensure survival, and maximum water volume/fish weight relationship (125 ml/g fish) to minimize transport costs.

other for dead fish) are fitted to the weight/length versus length data. This is a common pattern for several species, with implications for production/management/fisheries (e.g., Safran 1992).

Mortality data indicate that the maximum length of fish to be transported should range 6–8 cm for better survival. It should be noted, however, that some measures to decrease stress during transport (e.g. addition of buffers or cooling of the bags) may contribute to a higher survival rate of larger fish. Interestingly, 7.5–11.25 cm *A. saxatilis* are commonly advertised (e.g., http://www.freshmarine.com and http://www.bluezooaquatics.com, both accessed on July 1, 2016 and May 16, 2018). The suggestion of a size limit for the trade of a particular species is important to improve sustainable management of aquarium fisheries (Wabnitz et al. 2003).

The amount of water used for the transport of ornamental fish is important. Even though there is a tendency to use as little water as possible (to minimize transport costs), the survival and well-being of fish need to be ensured. Since the small fish in our experiment ranged between 4–6 cm, 70–80 ml water/g fish (< 6 cm) would ensure their survival according to our analysis. In order to guarantee a safety margin, we suggest a 125 mL water/g fish (or 8 g/l) to transport the small sergeant major fish (up to 6 cm) for up to 72 h with no mortality (drawn lines in Fig. 6).

Studies that address the effects of fish density during transport are more common for food fish. Normally, fish are transported in great numbers as fingerlings, packed inside large plastic bags. According to some studies, appropriate densities (species-specific values) for fish transport inside the bags are as follows: 300 g/l for *Brycon cephalus* (Günther, 1869) (Carneiro and Urbinati 2002), 206 g/l for *Brycon amazonicus* (Spix and Agassiz, 1829) (Abreu et al. 2008), 68 g/l for *Pimelodus maculatus* Lacepède, 1803 (Braun and Nuñer 2014), 78 g/l for *Colossoma macropomum* (Cuvier, 1816) (Gomes et al. 2003). High densities are preferred, in order to reduce costs, but as long as they do not increase mortality or morbidity rates. Interestingly, for *B. cephalus*, high densities can actually reduce stress due to reduced aggression and establishment of a structure of social dominance (Urbinati et al. 2004). However, as marine ornamentals are transported individually, studies that indicate best transport densities – in weight of fish/liter of water, or volume of water for a given length of fish – are relevant. Much lower densities in g/l of fish are needed for ornamentals, such as suggested here for *A. saxatilis*: 8g/l.



Figures 7–8. 3-D mesh plots showing the relationship among the 3 water parameters, whole set of data. NH_3-N (mg/L) versus DO(mg/L) versus pH, for live (7) and dead (8) fish. Legend illustrates color codes for pH interpolation.

Mortality data are actually the primary essential information in the development of protocols for the management of marine ornamental fish (Bruckner 2005). The causes of the high late mortality rates of marine ornamental fish are controversial (Rubec et al. 2001). The improvement in collection procedures, handling and transport tends to decrease mortality throughout the production chain (Bruckner 2005). The proposal of specific transport protocols for commercially relevant species can thus profit from the analysis of the causes of death of fish in transport simulation experiments.

Water parameters changing upon transport: what explains mortality?

The determination of a size limit or an adequate proportion between water volume and fish weight is relevant to establish a transport protocol for any given species. Nonetheless, it is also important to understand the reasons for fish mortality or morbidity during transport. Given that physiological parameters will obviously be altered in dead fish, it is interesting to ascertain if water parameters can give a clue on the reason for the deaths. If the proportion of water volume to fish mass is lower for larger fish – and it was, <75 mL/g – then the analysis of the water may give a hint.

Fish that died first (24 h) experienced acidosis (and acid water), and fish that died later (48 h) had water hypoxia. However, in either case, they produced less ammonia, and were much larger in size than the fish that survived. The transport times tested here were all "long", i.e., longer than eight hours (Davis 2006, Sampaio and Freire 2016). During these "long transport" times tested, both a reduction in pH and increase in ammonia were observed in live fish, consistent with the literature (Sampaio and Freire 2016). Given that mortality was clearly related to size, it was interesting to examine the relationship between each parameter and fish length, separately for live and dead fish. The only significant correlations to body length were those of water ammonia for the dead fish (-0.546, $p = 0.0433$), and water pH for the live fish (-0.512, $p = 0.0005$). This means that, the larger the size of the dead fish, the lower the ammonia in the water. This was not expected from the literature data, which often relates mortality to increased levels ammonia (Gomes et al. 2009, Tondolo et al. 2013, Parodi et al. 2014). Further, the longer the fish, the lower the pH of the water; that is, larger fish (in less water) acidify the transport water more.

Given that single parameters, when related to body length, were also not particularly conclusive, since larger fish that died produced less ammonia, these same three parameters (DO, NH_3 and pH) were again analyzed, but in a pairwise manner. In this analysis, Pearson correlations were calculated, also separately for live and dead fish. Significant correlations occurred only for live fish. There was a positive correlation between pH and DO (0.377, $p = 0.0139$), and a negative correlation between pH and ammonia (-0.473, $p = 0.0016$). DO drop follows pH decreases, and increased ammonia occurs concomitantly with pH drop for live fish. Over the hours of transport of *A. saxatilis*, the metabolism of fish that survived consumed oxygen (yields CO_2, which acidifies the water), NH_3 was putatively released (from protein metabolism, which alkalinizes the water); pH was observed to drop if there was higher input of CO_2 than NH_3 into the water (Sampaio and Freire 2016). Compatibly, as seen in Figs 1–3, ammonia levels increased in the water of dead fish, but less so than in live fish.

The situation of *A. saxatilis* seems to be of internal acidosis from CO_2/lactate production, resulting in water acidification (Sampaio and Freire 2016). Water acidification can result from metabolic CO_2 production and release to the water, whereas

lower NH_3 excretion may have resulted from metabolic depression from acid accumulation; intracellular low pH is suggested to trigger metabolic depression (Guppy et al. 1994). Metabolic depression is also consistent with dead fish showing lower ammonia levels, and variable DO in the water after 48 hours. Although DO levels were ≤2.1 mg/L in the water of four dead fish, for the other 10 dead fish DO levels remained between 2.5 and 8.5 mg/L. Acidosis may have led to later (48 hours) increased oxygen uptake, similar to the "excess post-exercise oxygen consumption"(Wood 1991), apparent in the water of the fish that died later, between 24 and 48 hours. Indeed, exhaustive exercise and severe stress are metabolically similar situations (Wood 1991, Milligan 1996, Kieffer 2000). This happens in all fish, but small fish have much more water/g of fish, so the effects are present in lower magnitude. In addition, larger fish need more time to recover from exhaustive exercise, and thus, potentially, from the initial stages of the stress of confinement inside the bag (Gingerich and Suski 2012).

The analysis in the three-dimensional graph ("mesh-plot") integrated the three water parameters, and more clearly showed the importance of the drop in pH to explain mortality of the largest fish. When the three water parameters were analyzed together, for live fish, it is apparent that the pH of the water decreased in association with a reduction in DO (Fig. 7). However, for the water of the dead fish, a "pH-trap" or "pH-valley" seems apparent (Fig. 8). Given the low volume of water available, most (large) fish died within 24 hours.

Fish and other aquatic animals that breath water do not accumulate CO_2 in the extracellular fluid and therefore do not have the bicarbonate buffer system of animals that breath air (Schmidt-Nielsen 1996, Hill et al. 2012). This is a result of the high solubility of CO_2 in water. Thus, it can be expected that the generation of lactate by anaerobic metabolism and/or CO_2 accumulation in the water released by fish entails metabolic acidosis, which ends up acidifying the water and causing fish death. More importantly, fish, especially those from coastal or intertidal habitats, display Hb with significant Bohr and Root effects. Through these effects, O_2 is efficiently delivered to tissues, as low pH reduces both affinity for Hb and the carrying capacity of blood oxygen (Rummer and Brauner 2015). Given their small size, it was not possible to measure lactate or blood pH, which would have been extremely relevant to confirm these hypotheses. For example, in cod fingerlings, *Gadus morhua* Linnaeus, 1758, water pH decreased sharply in a closed system (Treasurer 2012). In the case of the clownfish, *Amphiprion ocellaris* Cuvier, 1830, a relationship between a decrease in pH and mortality was noted at pH <6.3 (Chow et al. 1994). A transport simulation experiment with the giant miss, *Lates calcarifer* (Bloch, 1790), associated higher mortality to increased CO_2 in the water (Paterson et al. 2003). As a practical recommendation, we suggest in such cases the use of substances that have a buffering effect (Paterson et al. 2003, Treasurer 2012). However, this should be tested, as the buffer may worsen the condition of the transport water by facilitating the production and release of ammonia (Paterson et al. 2003, Treasurer 2012). A further test should involve the use of buffers along with ammonia quenchers (Chow et al. 1994).

Abudefduf saxatilis inhabits tide pools (Barreiros et al. 2004, Cunha et al. 2008, Freitas et al. 2009). Fish from such environmentally unstable habitats are more tolerant to variations in NH_3, DO, pH, salinity, and temperature (Richards 2011, Freire et al. 2011). Considering the three parameters measured in this study (DO, ammonia and pH), we conclude that 1) the release of ammonia in the package was not enough to cause mortality; 2) pure oxygen added to the shipping container, a procedure commonly used by traders, can prevent a hypoxia; 3) low pH was correlated with mortality (especially within the first 24 hours of transport, of larger fish). Further research, however, is required to elucidate if this is due to acidosis of the fish (lactate build up) and/or increased CO_2 in the water.

Physiological parameters

The analysis aimed to examine the relationship between mortality rate and water deterioration. However, it is also important to verify the concomitant physiological changes occurring in live *A. saxatilis* fish during long transport. In general, the physiological parameters measured in this study (osmolality, muscle water content, and glucose) remained stable in fish that survived the long transport simulations.

The non-significant variability in the values of plasma osmolality and muscle water content reflects the ability of fish to regulate extracellular osmolality and tissue water, typical of fish in general, especially in situations of unchanged water salinity, as they were transported in closed vials containing full strength seawater. This stability is even more likely in a marine group of teleosts related to unstable environments such as tide pools, as is the case of the Pomacentridae (Marshall and Grosell 2006, Evans and Claiborne 2009).

The high variability in blood glucose levels is probably a result of individual variations, potentialized by handling and confinement stress. Stress effects through cortisol could potentially affect glycogen and glucose metabolism in these fish (Mommsen et al. 1999). In addition, these fish were collected from the environment and were directly packaged, so it was not possible to know when they fed for the last time prior to being captured. Still, fasting for 24 h should bring glucose levels back to baseline. For better understanding of glycemia as a marker of transport stress, more research is needed to evaluate fed/fasted fish, followed by transport simulations (Mommsen et al. 1999, Sampaio and Freire 2016).

Plasma osmolality or glucose, and the activities of metabolic enzymes, are part of a secondary response to stress (see review in Sampaio and Freire 2016). The activity of the branchial carbonic anhydrase (CA) was not altered by the transport simulation conducted here, despite the evidence of acid release by fish into the water, resulting in a reduction of water pH, even in live fish. The branchial CA is very relevant for osmoregulation,

respiration, and acid base regulation (Marshall and Grosell 2006). The CA is a useful marker of exposure to pollutants, is sensitive to a wide variety of molecules (see Lionetto et al. 2012), but has not been used to assess metabolic disorders during fish transport.

Likewise, the enzymes of oxidative stress in fish are commonly used to measure the stress caused by drugs, heavy metals and other pollutants (Roche and Bogé 1996, Farombi et al. 2007). Studies to evaluate the stress of transport are recent, and use these markers also to verify the effects of using anesthetics during transport (e.g., Parodi et al. 2014, Salbego Joseânia 2014, Zeppenfeld et al. 2014). The specific activities of glutathione-S-transferase (GST) and catalase (CAT) were not different among the fish of the reference group and the three experimental groups (24, 48, and 72 h). However, the activity of the hepatic superoxide dismutase (SOD) decreased during the transport time. The decrease in SOD activity in transported fish can be related to a state of reduced metabolism in these fish, which is compatible with the stabilization in ammonia production rate and pH along time, apparent in Fig. 2, for live fish. Further research should be conducted within 24–72 hours of transport for a better understanding of the activation/suppression of the antioxidant response. For example, *Rhamdia quelen* (Quoy & Gaimard, 1824) transported for six hours in the presence of the essential oil of *Lippia alba* showed no change in liver SOD, but showed significant decrease in the activities of CAT and GST, compared to the control group (Salbego et al. 2014). With or without the use of anesthetics during transport, activation or inhibition of the antioxidant response should be more intensely studied, especially when fish reach their final destination. Special care should be taken when fish are transferred from their restricted transport vials, which may be hypoxic, to a stable aquarium, saturated with oxygen. Probably for that reason, marketers adopt protocols of acclimation of newly arrived fish: they drip water from the new system to the packaging of fish along periods of up to 4 hours. This procedure prevents sudden increases in dissolved oxygen which could foster the generation of oxygen free radicals.

Finally, marine ornamentals are sold according to size, which determines their market value. Conservatively, it is important to note that the maximum size of *A. saxatilis* for trade should be about 6 cm. For other species of MOF, the recommended maximum size for transport and appropriate relationships between water volume and fish weight should be established, in order to minimize costs, reduce stress and increase survival rates. Physiological and biochemical parameters evaluated in this study were not effective to characterize the stress of transport on individuals of this species. The assessment of changes in water quality during transport enabled a better understanding of the parameters that can affect fish homeostasis. With this approach, we propose that more studies be conducted on the physiology and water quality of the culture and transport of marine ornamental fish, aiming at their conservation. Ideally, more species should be cultivated, and successfully transported, to reduce their capture rates from nature.

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